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<p>(21) International Application Number: PCT/US99/20353</p> <p>(22) International Filing Date: 03 September 1999 (03.09.1999)</p> <p>(30) Priority Data: 09/259,240 26 February 1999 (26.02.1999) US 60/099,018 03 September 1998 (03.09.1998) US PCT/US99/04181 26 February 1999 (26.02.1999) US</p> <p>(60) Parent Application or Grant VENTANA MEDICAL SYSTEMS, INC. [/]; O. CHRISTENSEN, Kimberly [/]; O. MACREA, Ethel [/]; O. SEBASTIAO, Noemi [/]; O. CHRISTENSEN, Kimberly [/]; O. MACREA, Ethel [/]; O. SEBASTIAO, Noemi [/]; O. PENN, Amir, N. ; O.</p>		Published
<p>(54) Title: REMOVAL OF EMBEDDING MEDIA FROM BIOLOGICAL SAMPLES AND CELL CONDITIONING ON AUTOMATED STAINING INSTRUMENTS</p> <p>(54) Titre: EXTRACTION DES MILIEUX D'INCORPORATION D'ECHANTILLONS BIOLOGIQUES ET CONDITIONNEMENT CELLULAIRE SUR DES INSTRUMENTS DE COLORATION AUTOMATIQUE</p> <p>(57) Abstract</p> <p>The present invention provides an automated for removing or etching embedding media by exposing the biological samples for use in histochemical or cytochemical staining procedures without the dependence on organic solvents. The method comprises a biological sample slide in contact with a thermal platform, with or without heat, and with or without a fluid, to facilitate removal or etching of the embedding media from the biological sample. The present invention also provides an automated method for cell conditioning biological samples wherein the cells are predisposed for access by reagent molecules for histochemical and cytochemical staining procedures. The method comprises a biological sample slide in contact with a thermal platform, with or without heat, and with or without a fluid, to facilitate molecular access to cells and cell constituents within the biological sample.</p> <p>(57) Abrégé</p> <p>La présente invention concerne l'extraction ou l'élimination automatique de milieux d'incorporation consistant à exposer les échantillons biologiques destinés à être utilisés dans des procédures de coloration histochimique ou cytochimique sans dépendance vis-à-vis des solvants organiques. Le procédé comprend une lame d'échantillon biologique au contact d'une plate-forme thermique, avec ou sans chaleur, et avec ou sans fluide, pour faciliter l'extraction ou l'élimination des milieux d'incorporation de l'échantillon biologique. La présente invention concerne également un procédé automatique de conditionnement cellulaire d'échantillons biologiques dans lequel les cellules sont prédisposées pour permettre un accès à des molécules de réactif pour des procédures de coloration histochimique et cytochimique. Le procédé comprend une lame d'échantillon biologique au contact d'une plate-forme thermique, avec ou sans chaleur, et avec ou sans fluide, pour faciliter l'accès moléculaire aux cellules et aux constituants des cellules se trouvant dans l'échantillon biologique.</p>		

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<p>(54) Title: REMOVAL OF EMBEDDING MEDIA FROM BIOLOGICAL SAMPLES AND CELL CONDITIONING ON AUTOMATED STAINING INSTRUMENTS</p> <p>(57) Abstract</p> <p>The present invention provides an automated for removing or etching embedding media by <i>exposing</i> the biological samples for use in histochemical or cytochemical staining procedures without the dependence on organic solvents. The method comprises a biological sample slide in contact with a thermal platform, with or without heat, and with or without a fluid, to facilitate removal or etching of the embedding media from the biological sample. The present invention also provides an automated method for <i>cell conditioning</i> biological samples wherein the cells are predisposed for access by reagent molecules for histochemical and cytochemical staining procedures. The method comprises a biological sample slide in contact with a thermal platform, with or without heat, and with or without a fluid, to facilitate molecular access to cells and cell constituents within the biological sample.</p>			

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Description

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10 *Removal of Embedding Media from Biological Samples and Cell
Conditioning on Automated Staining Instruments*

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15 **REFERENCE TO RELATED APPLICATIONS**

5 This application claims priority benefits to U.S. Provisional Patent Application Serial No. 60/099,018 filed on September 3, 1998, entitled Removal of Embedding Media from Tissue Samples and Cell and Tissue Conditioning in Automated Immunohistochemical Instrumentation (which is hereby incorporated by reference). This application also claims priority benefits to U.S. Patent Application Serial No. 09/259,240
20 filed on February 26, 1999, entitled Automated Molecular Pathology Apparatus Having Independent Slide Heaters (which is hereby incorporated by reference). This application further claims priority benefits to PCT Patent Application Serial No. PCT/US99/04181 filed on February 26, 1999, entitled Automated Molecular Pathology Apparatus Having
25 Independent Slide Heaters (which is hereby incorporated by reference).

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30 **BACKGROUND OF THE INVENTION**

35 **Field of the Invention**

20 The present invention relates to a method for removing embedding media from biological samples on automated instruments prior to immunohistochemical (IHC), *in situ* hybridization (ISH) or other histochemical or cytochemical manipulations. The present invention also relates to a method for conditioning cells or tissues so as to increase the
35 accessibility of various molecules to their respective targets and generally to improve tissue and cell readability.

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45 **Summary of the Related Art**

40 Diagnosis of disease based on interpretation of tissue or cell samples taken from a diseased organism has expanded dramatically over the past few years. In addition to traditional histological staining techniques and immunohistochemical assays, *in situ* techniques such as *in situ* hybridization and *in situ* polymerase chain reaction are now used
50 to help diagnose disease states in humans. Thus, there are a variety of techniques that can assess not only cell morphology, but also the presence of specific macromolecules within cells and tissues. Each of these techniques requires the preparation of sample cells or

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10 tissues which may include fixing the sample with chemicals such as an aldehyde (such as formaldehyde, glutaraldehyde), formalin substitutes, alcohol (such as ethanol, methanol, isopropanol) or embedding the sample in inert materials such as paraffin, celloidin, agars, polymers, resins, cryogenic media or a variety of plastic embedding media (such as epoxy

15 resins and acrylics). Other sample tissue or cell preparations require physical manipulation such as freezing (frozen tissue section) or aspiration through a fine needle (fine needle aspiration (FNA)). Regardless of the tissue or cell sample or its method of preparation or preservation, the goal of the technologist is to obtain accurate, readable and reproducible results that permit the accurate interpretation of the data. One way to provide accurate,

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10 readable and reproducible data is to prepare the tissue or cells in a fashion that optimizes the results of the test regardless of the technique employed. In the case of immunohistochemistry and *in situ* techniques this means increasing the amount of signal obtained from the specific probe (antibody, DNA, RNA). In the case of histochemical staining it may mean increasing the intensity of the stain or increasing staining contrast.

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15 Without preservation, tissue samples rapidly deteriorate such that their use in diagnostics is compromised shortly after removal from their host. In 1893, Ferdinand Blum

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discovered that formaldehyde could be used to preserve or fix tissue so that this tissue could be used in histochemical procedures. The exact mechanisms by which formaldehyde acts in fixing tissues are not fully established, but they involve cross-linking of reactive sites

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20 within the same protein and between different proteins via methylene bridges (Fox et al., *J. Histochem. Cytochem.* 33: 845-853 (1985)). Recent evidence suggests that calcium ions also play a role (Morgan et al., *J. Path.* 174: 301-307 (1994)). These links cause changes in the quaternary and tertiary structures of proteins, but the primary and secondary structures

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40 appear to be preserved (Mason et al., *J. Histochem. Cytochem.* 39: 225-229 (1991)). The

45 extent to which the cross-linking reaction occurs depends on conditions such as the concentration of formalin, pH, temperature and length of fixation (Fox et al., *J. Histochem. Cytochem.* 33: 845-853 (1985)). Some antigens, such as gastrin, somatostatin and α -1-antitrypsin, may be detected after formalin fixation, but for many antigens, such as

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50 intermediate filaments and leukocyte markers, immunodetection after formalin treatment is lost or markedly reduced (McNicol & Richmond, *Histopathology* 32: 97-103 (1998)). Loss of antigen immunoreactivity is most noticeable at antigen epitopes that are discontinuous,

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10 *i.e.* amino acid sequences where the formation of the epitope depends on the confluence of portions of the protein sequence that are not contiguous.

15 Antigen Retrieval™ is a term that describes the attempt to "undo" the structural changes that treatment of tissue with a cross-linking agent induces in the antigens resident within that tissue. Although there are several theories that attempt to describe the mechanism of Antigen Retrieval™ such as loosening or breaking of crosslinkages formed by formalin fixation, it is clear that modification of protein structure by formalin is reversible under conditions such as high-temperature heating. It is clear that several factors 20 affect Antigen Retrieval™: heating, pH, molarity and metal ions in solution (Shi et al., *J. 10 Histochem. Cytochem.* **45**: 327-343 (1997)).

25 Microwave heating appears to be the most important factor for retrieval of antigens masked by formalin fixation. Microwave heating (100°±5°C) generally yields better results in Antigen Retrieval™ immunohistochemistry (AR-IHC).

30 Different heating methods have been described for *antigen retrieval* in IHC such as 15 autoclaving (Pons et al., *Appl. Immunohistochem.* **3**: 265-267 (1995); Bankfalvi et al., *J. Path.* **174**: 223-228 (1994)), pressure cooking (Miller & Estran, *Appl. Immunohistochem.* **3**: 190-193 (1995); Norton et al., *J. Path.* **173**: 371-379 (1994)); water bath (Kawai et al., *Path. Int.* **44**: 759-764 (1994)), microwaving plus plastic pressure cooking (U.S. Patent No.; 35 Taylor et al. (1995); Pertschuk et al., *J. Cell Biochem.* **19**(suppl.): 134-137 (1994)), and 20 steam heating (Pasha et al., *Lab. Invest.* **72**: 167A (1995); Taylor et al., *CAP Today* **9**: 16-22 (1995)).

40 Although some antigens yield satisfactory results when microwaving is performed in distilled water, many antigens require the use of buffers during the heating process. 45 Some antigens have particular pH requirements such that adequate results will only be achieved in a narrow pH range. Presently, most Antigen Retrieval™ solutions are used at a pH of approximately 6-8, but there is some indication that slightly more basic solutions may provide marginally superior results (Shi, et al., *J. Histochem. Cytochem.* **45**: 327-343 (1997)).

50 Although the chemical components of the Antigen Retrieval™ solution, including 30 metal ions, may play a role as a possible co-factor in the microwaving procedure, thus far,

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no single chemical has been identified that is both essential and best for Antigen RetrievalTM.

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Many solutions and methods are used routinely for staining enhancements. These may include but are not limited to distilled water, EDTA, urea, Tris, glycine, saline and citrate buffer. Solutions containing a variety of detergents (ionic or non-ionic surfactants) may also facilitate staining enhancement under a wide range of temperatures (from ambient to in excess of 100°C).

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In addition to cell surface molecules that may be present on the exterior portion of the cell, other molecules of interest in IHC, ISH and other histochemical and cytochemical

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manipulations are located within the cell, often on the nuclear envelope. Some of these molecules undergo molecular transformation when exposed to a fixative (coagulative or precipitative) such as formalin. Thus with respect to these molecules it is desirable to not only overcome the effects of fixation but also to increase the permeability of the cell in order to facilitate the interaction of organic and inorganic compounds with the cell.

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Other tissue samples may not have been subjected to cross-linking agents prior to testing, but improved results with respect to these tissues is also important. There are a variety of non-formalin methods for preserving and preparing cytological and histological samples. Examples of these methods include but are not limited to a) hematology smears, cytospinsTM, ThinPrepsTM, touch preps, cell lines, Ficoll separations for lymphocytes and buffy coats etc. are routinely preserved in a many ways which include but are not limited to air-drying, alcoholic fixation, spray fixatives and storage mediums such as sucrose/glycerin storage medium. b) tissues and cells (either fixed or unfixed) may be frozen and subsequently subjected to various stabilizing techniques such preservation, fixation and desiccation. c) tissues and cells may be stabilized in a number of non-cross-linking aldehyde fixatives, non- aldehyde containing fixatives, alcoholic fixatives, oxidizing agents, heavy metal fixatives, organic acids and transport media.

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One way to improve testing results is to increase the signal obtained from a given sample. In a general sense, increased signal can be obtained by increasing the accessibility of a given molecule for its target. As in the case for antigens found within the cell, targets within the cell can be made more accessible by increasing the permeability of the cell thereby permitting a greater number of molecules entry into the cell, thereby increasing the

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probability that the molecule will "find" its target. Such increased permeability is especially important for techniques such as ISH, *in situ* PCR, IHC, histochemistry and cytochemistry.

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Tissues and cells are also embedded in a variety of inert media (paraffin, celloidin, OCT™, agar, plastics or acrylics etc.) to help preserve them for future analysis. Many of these inert materials are hydrophobic and the reagents used for histological and cytological applications are predominantly hydrophilic, therefor the inert medium may need to be removed from the biological sample prior to testing. For example, paraffin embedded tissues sections are prepared for subsequent testing by removal of the paraffin from the tissue section by passing the slide through various organic solvents such as toluene, xylene, limonene or other suitable solvents. Traditional deparaffinization uses organic solvents, which generally requires that the process be performed in ventilated hoods. Furthermore use and disposal of these solvents increases the cost of analysis and exposure risk associated with each tissue sample tested.

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Presently, there is no available technique for removing inert media from sample tissue by directly heating the slide in an automated fashion. Neither is it currently possible to remove inert media from sample tissue while conditioning the sample tissue or cell in a one-step automated staining process.

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The methods of the present invention permit a) automated removal of embedding media without the use of organic solvents, thus *exposing* the cells for staining and thereby reducing time, cost and safety hazards b) automated *cell conditioning* without automated removal of embedding media from the sample cell or tissue. c) a multi-step automated process that *exposes* the cells, performs *cell conditioning* and increases permeability of the cytological or histological specimens, thereby increasing sample readability and improving interpretation of test data. The methods of the present invention can be used for improving the stainability and readability of most histological and cytological samples used in conjunction with cytological and histological staining techniques.

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SUMMARY OF THE INVENTION

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The present invention relates to an automated method for *exposing* biological samples for use in histological or cytological testing procedures by removing the embedding media without the use of organic solvents.

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The present invention further relates to an automated method for *cell conditioning*, thus improving the accessibility of molecules in biological samples.

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The present invention also relates to an automated method for the simultaneous *exposing* and *cell conditioning* of biological samples for histochemical and cytochemical applications.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

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One embodiment of the present invention relates to the *exposing* of biological samples by removal of the inert materials in which biological samples have been embedded for preservation and support. In a preferred embodiment of the present invention, paraffin or other inert materials are removed from biological samples by heating one side of the biological sample. This may be accomplished by contact heating of the microscope slide on which the embedded biological samples have been placed. Other inert materials that can be removed from embedded biological samples include but are not limited to agars and cryogenic media. This process of removal of inert embedding media or etching of embedding media is referred to herein as *exposing*.

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In a preferred method of the present invention, the paraffin-embedded biological sample laying on the glass slide is first heated by a heating element. The heating element exposes heat on one side of the biological sample (such as the thermal platforms disclosed in U.S. patent application 09/259,240, herein incorporated by reference) within an automated staining instrument (U.S. patent application Serial No. 08/995,052 filed on December 19, 1997 and U.S. provisional patent application Serial No. 60/076,198 filed on February 27, 1998, both of which are herein incorporated by reference) such that the sample slide is dried and the paraffin is melted. Typically, the biological sample is placed on a top surface of a slide (such as a glass slide). The slide

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10 is then placed on top of the thermal platform, so that the bottom surface of the slide is in contact with the thermal platform. The thermal platform, via conduction, heats the bottom portion of the slide. After the heating of the biological sample, the inert material may be removed from the slide by a fluid (as a gas or liquid). For example, the inert 5 material may be rinsed with DI water and a surfactant.

15 In another method of the present invention, a paraffin embedded biological sample is placed on a glass microscope slide and the microscope slide is placed on a heating element. A reagent is placed on the biological sample slide, the biological sample slide is then exposed to elevated temperatures that will permit the melting of the 20 inert material, and after which the inert material may be removed from the slide by a fluid (as a gas or liquid).

25 In a preferred embodiment of the present invention, reagents are used in conjunction with heating the embedded biological samples. Suitable reagents may include, but are not limited to, de-ionized water, citrate buffer (pH 6.0-8.0), Tris-HCl 15 buffer (pH 6-10), phosphate buffer (pH 6.0-8.0), SSC buffer, APK Wash™, acidic buffers or solutions (pH 1-6.9), basic buffers or solutions (pH 7.1-14), mineral oil, 30 Norpar, canola oil, and PAG oil. Each of these reagents may also contain ionic or non-ionic surfactants such as Triton X-100, Tween, Brij, saponin and sodium dodecylsulfate.

35 In a method of the present invention, the temperature of the heating element is raised to a temperature in excess of the melting point of the inert material. For example, 20 the melting point of *pure* paraffin is listed as 50-57° C in the Merck index. Thus, in the method of the present invention, the temperature is in excess of the melting point of the paraffin in which the biological sample is embedded. In a preferred method of the 40 present invention, the temperature is raised in excess of 50° C to about 130° C.

25 In a method of the present invention, the duration of time required to melt the inert material will vary according to the temperature used and the embedding material. 45 Typically, in an automated system, a processor, such as a microprocessor, is used in conjunction with a memory. The amount of time and the temperature required to melt the paraffin is contained within a table contained in the memory.

30 The paraffin embedded biological sample is subjected to elevated temperatures 50 ranging from 5 minutes to 60 minutes. The heating element used in the method of the

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present invention requires that sufficient contact be maintained between the surface on
10 which the biological sample is placed and the heating element.

Another embodiment of the present invention relates to the *exposing* of
15 biological samples without removal of the inert materials in which biological samples
have been embedded for preservation and support. In a preferred embodiment of the
20 present invention, biological samples are readied for testing by contact heating of the
microscope slide on which the embedded biological samples have been placed. Other
inert materials that are not removed from embedded biological samples include but are
not limited to plastic or celloidin embedding media and/or other polymers and resins.

25 10 In a preferred method of the present invention, the embedded biological sample
laying on the glass slide is first heated by the heating element. The heating element
exposes heat on one side of the biological sample, such as by using the thermal
30 platforms disclosed in U.S. patent application 09/259,240 within an automated staining
instrument (U.S. patent applications 08/995,052 and 60/076,198) such that the sample
slide is dried.

35 15 In another method of the present invention, an embedded biological sample is
placed on a glass microscope slide and the microscope slide is heated on one side (e.g.,
by placing the slide on a thermal platform). A reagent is then placed on the biological
40 sample slide and the biological sample slide, with the reagent, is then heated to a
20 specified temperature (ranging from ambient to greater than 100°C) and for a specified
amount of time (ranging from 2 minutes to 12 hours). This will cause etching of the
45 surface of the inert embedding material, and after which the etching reagent may be
removed from the slide by a fluid (as a gas or liquid). As discussed previously, the
amount of time and the specified temperature may be stored in memory.

50 25 In the preferred method of the present invention, reagents are used in
conjunction with or without heating the embedded biological samples. Suitable reagents
45 may include, but are not limited to, de-ionized water, citrate buffer (pH 6.0-8.0), Tris-
HCl buffer (pH 6-10), phosphate buffer (pH 6.0-8.0), SSC buffer, APK Wash™, acidic
55 buffers or solutions (pH 1-6.9), basic buffers or solutions (pH7.1-14) mineral oil,
30 Norpar, canola oil, and PAG oil. Each of these reagents may also contain ionic or non-
ionic surfactants such as Triton X-100, Tween, Brij, saponin and sodium dodecylsulfate.

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10 In the method of the present invention, the temperature of the heating element is set to an appropriate level for the drying or the etching of the embedded biological sample. For example, etching may be carried out with a basic solution of methanol sodium hydroxide (sodium methoxide) at temperatures ranging from ambient to 37°C.

15 5 In the method of the present invention, the duration of time required to etch the inert material will vary according to the temperature used and the embedding material (plastic or celloidin embedding media and/or other polymers and resins etc.). In a preferred method of the present invention the embedded biological sample is subjected to appropriate temperatures ranging from 2 minutes to 12 hours. The heating element
20 10 used in the method of the present invention requires that sufficient contact be maintained between the surface on which the biological sample is placed and the heating element.

25 25 A preferred embodiment present invention also comprises an automated method of *cell conditioning*, either concurrent with, subsequent to or independent of removal or
15 30 etching of the inert embedding material from the biological sample. Heating the biological sample in an appropriate (organic or inorganic) reagent has been found to improve the accessibility of the reagent to the target molecule in the cell (protein, nucleic acid, carbohydrate, lipid, pigment or other small molecule). This process of improving accessibility of the reagent (organic or inorganic) to the molecular target is
35 40 referred to herein as *cell conditioning*.

45 45 In one method of the present invention, *cell conditioning* is accomplished while the biological sample is being *exposed* as described above. In this method of the present invention, a biological sample is placed on a glass microscope slide and the microscope slide is heated on one side (e.g., by placing the slide on a thermal platform) within an
50 50 automated staining instrument (U.S. patent applications 08/995,052 and 60/076,198). A reagent is placed on the biological sample and the temperature of the heating element may or may not be increased. The biological sample is exposed to the appropriate temperature for an appropriate duration of time that will permit the melting or etching of the inert material and permit *cell conditioning* of the biological sample to be
55 30 subsequently stained using histological or cytological techniques.

10 The reagents used for *cell conditioning* can be the same as those for *exposing* the embedded biological sample. For example, for DNA targets, a *cell conditioning* solution may be a solution of EDTA; a common temperature setting may be 95°C for a duration ranging from 6-60 minutes. For protein targets, a *cell conditioning* solution 15 may be a solution of boric acid buffer; a common temperature setting may be in excess of 100°C for a duration ranging from 6-60 minutes. For RNA targets, a *cell conditioning* solution may be a solution of SSC; a common temperature setting may be 75°C for a duration ranging from 6-60 minutes. For histochemical reactions, such as a 20 Hematoxylin and Eosin (H&E) stain, a *cell conditioning* solution may be treated de-ionized water; a common temperature may range from 60-80°C for a duration of 6-30 minutes. A partial list of possible reagents appears in *Analytical Morphology*, Gu, ed., 25 Eaton Publishing Co. (1997) at pp. 1-40. The solutions should generally be of known molarity, pH, and composition. Sodium dodecyl sulfate (SDS), ethylene glycol is preferably added to the conditioning solution. In addition, metal ions or other materials 15 may be added to these reagents to increase effectiveness of the cell conditioning.

30 In another method of the present invention, *cell conditioning* is accomplished 35 subsequent to the biological sample being *exposed* as described above. In this method of the present invention a biological sample is placed on a glass microscope slide and the microscope slide is heated on one side (e.g., by placing the slide on a thermal 40 platform) within an automated staining instrument (U.S. patent applications 08/995,052 and 60/076,198). In this method, the embedded biological sample laying on the glass slide is first heated by the heating element within an automated staining instrument such that the sample slide is dried and the embedding material is melted or etched and 45 removed by the application of a fluid. Subsequent to *exposing* the biological sample, an 50 appropriate reagent is applied in order to permit *cell conditioning* of the biological sample to be subsequently stained using histological or cytological techniques.

45 The reagents used for *cell conditioning* can be the same as those for *exposing* 55 the embedded biological sample. For example, for DNA targets, a *cell conditioning* solution may be a solution of SSC; a common temperature setting may be 95°C for a duration ranging from 6-60 minutes. For protein targets, a *cell conditioning* solution may be a solution of Phosphate buffer; a common temperature setting may be in excess

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of 100°C for a duration ranging from 6-60 minutes. For RNA targets, a *cell conditioning* solution may be a solution of SSC; a common temperature setting may be 75°C for a duration ranging from 6-60 minutes. For histochemical reactions, such as a Trichrome stain, a *cell conditioning* solution may be Bouins; a common temperature 5 may range from 60-80°C for a duration of 6-30 minutes.

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In yet another method of the present invention, *cell conditioning* is accomplished without the biological sample being *exposed*. In this method of the present invention, a biological sample is placed on a glass microscope slide and the microscope slide placed on a heating element within an automated staining instrument.

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10 A reagent is placed on the biological sample and the temperature of the heating element may or may not be increased. *Cell conditioning* of the biological sample may be performed prior to being stained using histological or cytological techniques.

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25 The reagents used for *cell conditioning* can be the same as those for *exposing* the embedded biological sample. For example, for DNA targets, a *cell conditioning* 15 solution may be a solution of Sodium Citrate; a common temperature setting may be 90°C for a duration ranging from 6-60 minutes. For protein targets, a *cell conditioning* solution may be a solution of urea; a common temperature setting may be in excess of 30 100°C for a duration ranging from 6-60 minutes. For whole cells, a *cell conditioning* solution may be a solution of methanol; a common temperature setting may be ambient 35 20 for a duration ranging from 4-10 minutes. For histochemical reactions, such as an Acid Fast Bacilli (AFB) stain, a *cell conditioning* solution may be peanut oil; a common temperature may range from 60-70°C for a duration of 30-60 minutes.

40

The present invention also comprises *cell conditioning* of cytological preps, such as fine needle aspirations (FNA) smears, touch preps, Ficoll, Cytospins®, Thins 45 25 Preps®, cervical-vaginal pap smears, blood or body fluid films, etc., that are neither fixed with an aldehyde nor embedded in a matrix, such as paraffin. Many are fixed in an alcohol, such as methanol or ethanol, others will be sprayed with hair spray or other aerosol fixative and dried, and still others will be placed in cytological fixatives, which may include carbowax and Saccomanno's (organic or inorganic) reagent among others. 30 30 The cells are either centrifuged or filtered to a slide or directly touched to a glass slide and smeared in some cases (PAP's) or applied directly against the slide (touch preps).

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10 The term "Biological sample" is meant any collection of cells (either loose or in tissue) that can be mounted on a standard glass microscope slide including, without limitation, sections of organs, tumors sections, bodily fluids, smears, frozen sections, blood, cytology preps, microorganisms and cell lines.

15 5 The term "Stain" is meant any biological or chemical entity which, when applied to targeted molecules in biological sample, renders the molecules detectable under microscopic examination. Stains include without limitation detectable nucleic acid probes, antibodies, and other reagents which in combination or by themselves result in a colored end product (by bright field or fluorescence).

20 10 The following examples are presented for illustrative purposes only and are not intended, nor should they be construed, as limiting the invention in any way. Those skilled in the art will recognize that variations on the following can be made without exceeding the spirit or scope of the invention. All patents, patent applications, and other 25 publications are hereby incorporated by reference in their entirety.

15 EXAMPLES

30 Example 1

Automated "Exposing" and "Cell Conditioning" with Biological Samples Stained with H&E

35 20 Biological samples, including breast CHTN33, stomach 149G, brain, tonsil and kidney, that had been embedded in paraffin were *exposed* according to the following procedure: slides containing the above referenced biological sample were placed on an automated instrument (Ventana Medical Systems, Inc., Tucson, AZ) and subjected to the *exposing* protocol described below. Generally, the slides containing paraffin 40 embedded biological samples were dry heated to 65° C for six (6) minutes then rinsed 45 25 with 1x citrate buffer, de-ionized water, 10mM phosphate buffer (pH = 6.3), or 10mM Tris-HCl buffer (pH = 7.4) each containing 0.1% Triton X-100.

45 *Exposing Protocol 1*

1. Incubate for 2 minutes
- 30 2. Rinse slide
- 50 3. Adjust slide volume and apply liquid coverslip™

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4. Incubate for 6 minutes
5. Rinse slide
6. Adjust slide volume and apply liquid coverslip™
7. Increase temperature to 65.0° C

5

8. Rinse slide
9. Adjust slide volume and apply liquid coverslip™
10. Incubate for 4 minutes
11. Adjust slide volume and apply liquid coverslip™
12. Incubate for 4 minutes

20

- 10 13. Adjust slide volume and apply liquid coverslip™
14. Incubate for 4 minutes
15. Rinse slide
- 25 16. Decrease temperature to 42.0° C
17. Adjust slide volume and apply liquid coverslip™
- 15 18. Incubate for 4 minutes
19. Rinse slide
- 30 20. Decrease temperature to 42.0° C
21. Adjust slide volume and apply liquid coverslip™
22. Incubate for 4 minutes
- 35 20 23. Rinse slide

After automated exposing, the biological sample was stained with hematoxylin and eosin by the following method. Slides were placed in hematoxylin 1 (Richard Allen Scientific, Kalamazoo, MI) for 1.5 minutes and then rinsed with running de-ionized water for one minute. Slides were then placed in acid alcohol clarifier (Richard Allen Scientific) for one minute and then rinsed with running de-ionized water for one minute. Slides were then placed in diluting ammonia-bluing reagent for one minute (Richard Allen Scientific, Kalamazoo, MI) and then rinsed in running de-ionized water for one minute. Slides were then rinsed in 95% ethanol, and then placed in 2.5% eosin Y (Richard Allen Scientific, Kalamazoo, MI) for 2.5 minutes. The biological samples on the slides were dehydrated by exposing the biological sample to a 100% ethanol bath for

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one minute. This process was repeated three times followed by exposure of the biological sample to a xylene bath for three minutes, twice. After the dehydration step the biological sample was covered with a coverslip.

15

Control biological samples were deparaffinized by a traditional solvent-based deparaffinization technique. Biological sample place on microscope slides and preserved in paraffin were completely submersed in a xylene bath for five minutes. Slides containing biological sample were placed in a second xylene bath for five minutes. After removal from the second xylene bath, the slides were placed in a 100% ethanol bath for three minutes. Slides were then placed in a second 100% ethanol bath for three minutes and then placed in a 90% ethanol solution for two minutes. The slides were then placed in 80% ethanol for one minute followed by complete immersion in distilled water for one to three minutes. After deparaffinization, the biological samples were stained with hematoxylin and eosin as described above.

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The biological samples that were deparaffinized by the solvent technique and by the automated heating technique were compared after staining by hematoxylin and eosin. Morphology on all sets of slide was acceptable and essentially equivalent. The tonsil and brain biological samples that were *exposed* by the automated heating method showed more intensified hematoxylin staining than the biological samples deparaffinized by standard solvent techniques.

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Example 2

Automated "Exposing" of Biological e Samples

with Simultaneous "Cell Conditioning"

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Biological samples of kidney Q-10 and tonsil T998D that had been formalin fixed and embedded in paraffin were *exposed* according to the protocol described in Example 1. After automated *exposing*, the biological sample was subjected to the DAB paraffin protocol used for immunohistochemical staining. The protocol for DAB staining is described below:

50

- 30 1. Incubate for 2 minutes
2. Rinse slide

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3. Adjust slide volume and apply liquid coverslip™

15

4. Rinse slide

5. Adjust slide volume and apply liquid coverslip™

6. Rinse slide

5

7. Adjust slide volume and apply liquid coverslip™

10

8. Apply one drop of inhibitor

15

9. Incubate for 4 minutes

10. Adjust slide volume and apply liquid coverslip™

20

11. Apply one drop of primary antibody

10

12. Incubate for 32 minutes

13. Adjust slide volume and apply liquid coverslip™

25

14. Apply one drop of Biotinylated Ig

15. Incubate for 8 minutes

16. Rinse slide

15

17. Adjust slide volume and apply liquid coverslip™

30

18. Apply one drop of Avidin-HRPO

19. Incubate for 8 minutes

20. Rinse slide

21. Adjust slide volume and apply liquid coverslip™

35

22. Apply one drop of DAB and one drop DAB H₂O₂

23. Incubate for 8 minutes

24. Rinse slide

25. Adjust slide volume and apply liquid coverslip™

40

26. Apply one drop of Copper

25

27. Incubate for 4 minutes

28. Rinse slide

45

The primary antibody used for the kidney Q-10 biological sample was Anti-CD15 (Ventana Medical Systems, Inc. Tucson, AZ, Catalogue no. 250-2504). The primary antibody used for the tonsil T998D biological sample was Anti-CD45RO (Ventana Medical Systems, Inc. Tucson, AZ, Catalogue no. 250-2563). The DAB

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staining kit used was obtained from Ventana Medical Systems, Inc. Tucson, AZ, Catalogue no. 250-001.

10

Control biological samples were deparaffinized by a traditional solvent-based deparaffinization technique, as described in Example 1. After deparaffinization the 5 biological samples were placed in a pressure cooker (Model #62104 Nordic Ware, Minneapolis, MN) containing 1.5 L 1x citrate buffer. The pressure cooker was then sealed and placed in a microwave oven (Model #MQSO836E, Matsushita, Franklin Park, IL). With the microwave oven set on "high," the samples were subjected to 15 microwave heating for approximately 30 minutes. After microwaving the samples were then "cured" for 30 minutes in the pressure cooker with the lid securely fastened. After 20 curing the biological samples were placed in 1x citrate buffer for two minutes. The biological samples were then removed from the citrate buffer and the end of the slides 25 blotted to remove excess citrate buffer. After blotting, the slides were placed on the automated instrument and immunohistochemically stained as described above.

25

30

15 The biological samples deparaffinized by the solvent technique and by the 30 automated *exposing* and simultaneous *cell conditioning* technique were compared after immunohistochemical staining. Morphology on all sets of slide was acceptable and essentially equivalent.

35

20 Example 3

Two Step Automated "Exposing" and "Cell Conditioning"

40

Biological sample samples of tonsil T998D, tonsil Ki67, E68, E7, E33, E8, E29, E15, and E68 that had been preserved in paraffin and treated with formaldehyde were treated by the following protocol:

45

25 **Exposing and Cell Conditioning**

50

1. Incubate for 2 minutes
2. Increase thermofoil temperature to 65.0° C
3. Incubate for 6 minutes
4. Rinse slide and apply coverslip
5. Incubate for 6 minutes
6. Rinse slide and apply coverslip

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7. Increase thermofoil temperature to 100.0° C
8. Adjust slide volume and apply liquid coverslip™
9. Rinse slide
10. Adjust slide volume and apply liquid coverslip™
11. Incubate for 4 minutes
12. Adjust slide volume and apply liquid coverslip™
13. Incubate for 4 minutes
14. Adjust slide volume and apply liquid coverslip™
15. Incubate for 4 minutes
16. Adjust slide volume and apply liquid coverslip™
17. Incubate for 4 minutes
18. Adjust slide volume and apply liquid coverslip™
19. Incubate for 4 minutes
20. Adjust slide volume and apply liquid coverslip™
21. Incubate for 4 minutes
22. Adjust slide volume and apply liquid coverslip™
23. Incubate for 4 minutes
24. Adjust slide volume and apply liquid coverslip™
25. Incubate for 4 minutes
26. Adjust slide volume and apply liquid coverslip™
27. Incubate for 4 minutes
28. Rinse slide
29. Decrease temperature to 42.0° C
30. Adjust slide volume and apply liquid coverslip™
31. Incubate for 4 minutes
32. Rinse slide
33. Decrease temperature to 20.0° C
34. Adjust slide volume and apply liquid coverslip™
35. Incubate for 4 minutes
36. Rinse slide

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10 The buffer used in the protocol was SSC buffer with either 20% formamide or 0.1% Triton. After the biological sample was subjected to the above protocol, the DAB paraffin protocol used for immunohistochemical staining of Example 2 was applied. Tonsil biological sample was treated with anti-Ki67 as a primary antibody. Samples

15 5 E68, E7, E33, and E8 biological sample was treated with anti-estrogen receptor (6F11) as a primary antibody. E29, E15, and E68 biological sample was treated with anti-progesterone receptor (1A6) as a primary antibody.

20 Control biological samples were deparaffinized by a traditional solvent-based de-paraffinization technique, as described in Example 1. After deparaffinization the 10 biological samples were placed in a pressure cooker (Model #62104 Nordic Ware, Minneapolis, MN) containing 1.5 L 1x citrate buffer. The pressure cooker was then sealed and placed in a microwave oven (Model #MQSO836E, Matsushita, Franklin 25 Park, IL). With the microwave oven set on "high", the samples were subjected to microwave heating for approximately 30 minutes. After microwaving the samples were 15 then "cured" for 30 minutes in the pressure cooker with the lid securely fastened. After curing the biological samples were placed in 1x citrate buffer for two minutes. The 30 biological samples were then removed from the citrate buffer and the end of the slides were blotted to removed excess citrate buffer. After blotting the slide were placed on the automated instrument and immunohistochemically stained as described above.

35 20 The biological samples deparaffinized by solvent technique and by the automated heating technique were compared after immunohistochemical staining. Morphology on all sets of slide was acceptable and essentially equivalent.

40

Example 4

25 *Automated Cell Conditioning of Non Paraffin Embedded Cell Lines for in situ
(Thin Preps™)*

45 Hela (lot # 980427H), Caski (lot # 980416C) and Siha (lot # 980416S) cell lines stored in Cytyk preparation solution (lot # 01139Q) were deposited on microscope 30 slides using the Cytyk 2000 instrument. After deposition the slides were placed in alcohol to keep moist until use on the Discovery® In-Situ staining module (Ventana 50 Medical Systems Inc., Tucson, AZ). Slides were loaded into the instrument and wetted

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with 2 X SSC made from 20 X SSC (Ventana P/N 650-012). Slides were run through a *cell conditioning* protocol currently referred to as Depar 30 where the slides are rinsed with 2 X SSC and the temperature of the slides is increased to 95° C for a period of approximately 30 minutes. The slides are then cooled to 37° C and rinsed with APK

5 Wash® prior to the in-situ staining run.

15

Using the protocol Blue Swap ISH the cell lines were stained for HPV 16/18 (Enzo HPV 16/18 Bio Probe cat # 32874). Prior to probe application the cell lines are enzymatically digested with Protease 2 (Ventana P/N 250-2019). After the probe application the probe and biological sample are denatured simultaneously at 95° C for 8 minutes. The non-specifically bound probe is washed off with stringency washes of 2 X SSC at 55° C. The probe is then detected with Streptavidin Alk Phos and NBT/BCIP.

20

The cell lines were dehydrated after staining with a one-minute exposure to 95 % ethanol and a one-minute exposure to 100% ethanol repeated 2 times. Following the ethanol the slides were exposed to xylene for 3 minutes twice. After dehydration the 15 slides were coverslipped.

30

The stained cell lines after conditioning showed acceptable morphology, there was high background on these slides indicating a need for the process to be developed more.

Wet Load Slides

35

- 20 1. Skip Application & Incubate for 2 minutes
2. Rinse Slides (2X SSC Buffer) (Warm Slides to 65° C)
3. Adjust Slide Volume, then Apply Coverslip
4. Skip Application & Incubate 6 minutes
- 40 5. Rinse Slides (2X SSC Buffer) (Warm Slides to 95° C)
- 25 6. Adjust Slide Volume, then Apply Coverslip
7. Rinse Slides
- 45 8. Adjust Slide Volume, then Apply Coverslip
9. Skip Application & Incubate for 4 minutes
10. Adjust Slide Volume, then Apply Coverslip
- 30 11. Skip Application & Incubate for 4 minutes
- 50 12. Adjust Slide Volume, then Apply Coverslip

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13. Skip Application & Incubate for 4 minutes
14. Adjust Slide Volume, then Apply Coverslip
15. Skip Application & Incubate for 4 minutes
16. Adjust Slide Volume, then Apply Coverslip
- 5 17. Skip Application & Incubate for 4 minutes
18. Adjust Slide Volume, then Apply Coverslip
19. Skip Application & Incubate for 4 minutes
20. Adjust Slide Volume, then Apply Coverslip
21. Skip Application & Incubate for 4 minutes
- 20 10 22. Rinse Slides (2X SSC Buffer) (Warm Slides to 37° C)
23. Adjust Slide Volume, then Apply Coverslip
24. Skip Application & Incubate for 4 minutes
- 25 25. Rinse Slides (APK Wash)
26. Adjust Slide Volume, then Apply Coverslip

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Example 5

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Automated "Exposing" and "Cell Conditioning" for single copy DNA detection

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Slides containing formalin fixed, paraffin embedded cell lines Caski (R96-1050A) and Siha (R96-96-C2) were stained on Ventana target slides. Slides were dry loaded onto the instrument and the slide temperature was increased to 65° C. Depar 30 protocol was run where the slides are rinsed with 2x SSC Buffer while at 65° C then the heat is increased to 95° C for about 40 minutes. The slides were then cooled to 37° C and rinsed with APK wash. At this time the following In Situ protocol was run:

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In-Situ Protocol: Tubbs 1

25 (Dako TBST # 3306 is substituted for Ventana APK Wash during non-probe steps)

45

Protease Digestion: Protease 2, 4 minutes, 37 °C

Inhibitor Step: Ventana Inhibitor from DAB kit 32 minutes 37 °C

Probe: Enzo HPV Bio Probe 16/18

30 Control Probe: Enzo HPV Bio Probe 6/11

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Denaturation: 95°C, 8 minutes

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Hybridization: 37°C, 64 minutes

2 Stringency Washes 2XSSC, 60°C, 8 minutes each

3rd Stringency Wash 2XSSC, 37°C, 4 minutes

Probe Detection: Streptavidin HRPO (Dako GenPoint #K0620)

5 Amplification: Biotinyl Tyramide (Dako GenPoint # K0620)

15

Detection: Streptavidin HRPO (Dako GenPoint #K0620)

or

Streptavidin Alk Phos (Vector # SA5100)

20

Chromogen DAB (Dako Gen Point # K0620)

10 or

Ventana NBT/BCIP (Kit P/N)

or

25

Ventana Naphthol / Fast Red (Kit P/N)

15

Example 6

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Automated "Cell Conditioning" for Non-paraffin Embedded Samples

The protocol for DAB staining as described in Example 2 was used in this Example.

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The *cell conditioning* steps for these antibodies was done after using a Cytyk 2000® instrument to make ThinPreps® of cell lines. The ThinPreps® were stained using antibodies to ER, PgR, Ki67, P53 on Ventana ES instruments, NexES instruments and a manual procedure (Cytyk, Inc.). A duplicate group of slides have been stained on the NesES Insitu module, allowing the *cell conditioning* steps to be performed by automation.

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25 Although the example stated above is specific to the Cytyk® instrument and staining of the ThinPreps®, the experience is not limited to that mode of making cytological preps.

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Example 7*"Cell conditioning" of frozen biological sample*

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Frozen tonsil blocks 297C and 297D were prepared by cutting six sections from each block and placing the sample on microscope slides. Four slides from each block 5 were placed on the Discovery™ Insitu module and put through protocol Depar 10.

15

Slides are dry heated to 65° for 6 minutes then rinsed with 0.1M EDTA buffer pH 8. After rinsing, the slide is incubated at 65° for 20 minutes. Slides were then cooled to 37° C and rinsed with APK Wash. Two slides from each block were left untreated as controls. Following the Depar 10 treatment two treated slides from each 20 block and one untreated slide were stained for H & E as described in Example 1. Two treated slides from each block and one untreated from each block are stained for LCA. Run outcomes: for both the H & E and antibody staining there was no staining 25 difference between the treated and untreated slides.

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From the foregoing detailed description, it will be appreciated that numerous 30 changes and modifications can be made to the aspects of the invention without departure from the true spirit and scope of the invention. This true spirit and scope of the invention is defined by the appended claims, to be interpreted in light of the foregoing specification.

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Claims

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What is claimed is:

10 1. An automated method of removal of embedding media from a biological sample within a biological staining procedure, the method comprising the steps of:

15 applying exposing reagents to the biological sample;

5 applying heat to the biological sample; and

15 applying fluid to remove the exposing reagents.

20 2. An automated method according to claim 1 wherein the biological sample is placed on a top side of a slide and wherein the step of applying heat to the biological sample includes exposing heat to a bottom side of the slide.

25 10 3. An automated method according to claim 2 wherein the bottom side of the slide is in contact with a thermal platform and wherein the step of exposing heat to a bottom side of the slide includes heating the slide by conduction using the thermal platform.

25 4. An automated method according to claim 1 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 130 °C.

30 15 5. An automated method according to claim 1 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 100 °C.

35 6. An automated method according to claim 1 wherein the step of applying fluid to remove the exposing reagents includes rinsing the slide with a rinsing medium.

7. An automated method according to claim 6 wherein the rinsing medium is

40 20 selected from the group consisting of air, de-ionized water, citrate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 6-10), phosphate buffer (pH 6.0-8.0), SSC buffer, APK Wash™, acidic buffers or solutions (pH 1-6.9), basic buffers or solutions (pH 7.1-14), mineral oil, Norpar, canola oil, and PAG oil.

45 40 8. An automated method according to claim 6 wherein the rinsing medium includes 25 ionic or non-ionic surfactants.

45 9. An automated method according to claim 8 wherein the ionic or non-ionic 45 surfactants are selected from the group consisting of Triton X-100, Tween, Brij, sodium dodecylsulfate and saponin.

50 10. An automated method of removing embedding media from a biological sample 30 within a biological staining procedure, the method comprising the steps of:

50 applying heat to the biological sample; and

applying fluid to remove the embedding media.

10 11. An automated method according to claim 10 wherein the biological sample is placed on a top side of a slide and wherein the step of applying heat to the biological sample includes exposing heat to a bottom side of the slide.

15 12. An automated method according to claim 11 wherein the bottom side of the slide is in contact with a thermal platform and wherein the step of exposing heat to a bottom side of the slide includes heating the slide by conduction using the thermal platform.

20 13. An automated method according to claim 10 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 130 °C.

25 14. An automated method according to claim 10 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 100 °C.

15 15. An automated method according to claim 10 wherein the fluid to remove the embedding media includes a detergent.

30 16. An automated method according to claim 10 further comprises the step of applying a second fluid prior to the step of applying heat to the biological sample.

15 17. An automated method according to claim 16 wherein the second fluid is de-ionized water.

35 18. An automated method for etching embedding media in a biological sample within a biological staining procedure, the method comprising the steps of:

20 19. 20. applying at least one etching solution; and
applying fluid to remove the at least one etching solution.

40 21. An automated method according to claim 18 further comprising the step of applying heat to the biological sample after the step of applying at least one etching solution.

45 22. An automated method according to claim 19 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 100 °C.

21. An automated method according to claim 1 wherein the step of applying fluid to remove the at least one etching solution includes rinsing the slide with a rinsing medium.

50 23. An automated method according to claim 21 wherein the rinsing medium is selected from the group consisting of air, de-ionized water, citrate buffer (pH 6.0-8.0),

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10 Tris-HCl buffer (pH 6-10), phosphate buffer (pH 6.0-8.0), SSC buffer, APK Wash™, acidic buffers or solutions (pH 1-6.9), basic buffers or solutions (pH 7.1-14), mineral oil, Norpar, canola oil, and PAG oil.

15 23. An automated method according to claim 21 wherein the rinsing medium includes ionic or non-ionic surfactants.

20 24. An automated method according to claim 23 wherein the ionic or non-ionic surfactants are selected from the group consisting of Triton X-100, Tween, Brij, sodium dodecylsulfate and saponin.

25 25. An automated method of cell conditioning without the removal or etching of the embedding media within a biological staining procedure, the method comprises the steps of:

26 applying heat to the biological sample;

27 applying at least one conditioning reagent; and

28 applying fluid to remove the at least one conditioning reagent.

30 29. An automated method according to claim 25 wherein the biological sample is heated to temperatures ranging from ambient to 130° C.

35 30. An automated method according to claim 25 wherein the at least one conditioning reagent is selected from the group consisting of air, de-ionized water, citrate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 6-10), phosphate buffer (pH 6.0-8.0),

35 31. SSC buffer, APK Wash™, acidic buffers or solutions (pH 1-6.9), basic buffers or solutions (pH 7.1-14) mineral oil, Norpar, canola oil, and PAG oil.

40 32. An automated method according to claim 25 wherein the at least one conditioning reagent contains ionic or non-ionic surfactants selected from the group consisting of Triton X-100, Tween, Brij, sodium dodecylsulfate and saponin.

45 33. An automated method of simultaneously removing embedding medium from a biological sample while providing cell conditioning within a biological staining procedure, the method comprising the steps of:

34 applying exposing and cell conditioning reagents;

35 applying heat to the biological sample;

36 applying fluid to remove the exposing and cell conditioning reagents; and

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37 staining the biological sample.

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10 30. An automated method according to claim 29 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 130 °C.

15 31. An automated method according to claim 29 wherein the exposing and cell conditioning reagents are selected from the group consisting of air, de-ionized water, citrate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 6-10), phosphate buffer (pH 6.0-8.0), SSC buffer, APK Wash™, acidic buffers or solutions (pH 1-6.9), basic buffers or solutions (pH7.1-14) mineral oil, Norpar, canola oil, and PAG oil.

20 32. An automated method according to claim 29 wherein the exposing and cell conditioning reagents contain ionic or non-ionic surfactants selected from the group consisting of Triton X-100, Tween, Brij, sodium dodecylsulfate and saponin.

25 33. An automated method of removing or etching embedding media from a biological sample and subsequently providing cell conditioning within a biological staining procedure, the method comprising the steps of:

30 applying heat to the biological sample;

35 15 applying a first fluid to the biological sample to remove the embedding media or etching reagents;

40 30 applying cell conditioning reagents;

45 35 applying a second fluid to remove the cell conditioning reagents; and staining of the biological sample.

50 20 34. An automated method according to claim 33 wherein the step of applying heat includes heating the biological sample to temperatures ranging from ambient to 130 °C.

55 35. An automated method according to claim 33 further comprising the step of applying exposing reagents to the biological sample

60 36. An automated method according to claim 35 wherein the exposing reagents are selected from the group consisting of air, de-ionized water, citrate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 6-10), phosphate buffer (pH 6.0-8.0), SSC buffer, APK Wash™, acidic buffers or solutions (pH 1-6.9), basic buffers or solutions (pH7.1-14) mineral oil, Norpar, canola oil, and PAG oil.

65 37. An automated method according to claim 35 wherein the exposing reagents contain ionic or non-ionic surfactants selected from the group consisting of Triton X-100, Tween, Brij, sodium dodecylsulfate and saponin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20353

A. CLASSIFICATION OF SUBJECT MATTER		
IPC(6) : G01N 1/30 US CL : 436/43, 46, 174, 175; 422/63, 66 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) U.S. : 436/43, 46, 174, 175; 422/63, 66		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,273,905 A (MULLER et al.) 28 December 1993 (28-12-93), Summary of the Invention, columns 8-11 and Embodiments section starting from column 68.	1-37
Y	US 4,043,292 A (ROGERS et al.) 23 August 1977 (23-08-77), whole document.	1-37
Y	US 5,023,187 A (KOEBLER et al.) 11 June 1991 (11-06-91), whole document.	1-37
Y	US 5,614,376 A (COPELEY et al.) 25 March 1997 (25-03-97), whole document.	1-37
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *B* earlier document published on or after the international filing date *L* documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 03 DECEMBER 1999		Date of mailing of the international search report 21 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Long V. Le</i> LONG V. LE Telephone No. (703) 308-0651

INTERNATIONAL SEARCH REPORT

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PCT/US99/20353

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 1/30

US CL :436/43, 46, 174, 175; 422/63, 66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/43, 46, 174, 175; 422/63, 66

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,273,905 A (MULLER et al.) 28 December 1993 (28-12-93), Summary of the Invention, columns 8-11 and Embodiments section starting from column 68.	1-37
Y	US 4,043,292 A (ROGERS et al.) 23 August 1977 (23-08-77), whole document.	1-37
Y	US 5,023,187 A (KOEBLER et al.) 11 June 1991 (11-06-91), whole document.	1-37
Y	US 5,614,376 A (COPELEY et al.) 25 March 1997 (25-03-97), whole document.	1-37

Further documents are listed in the continuation of Box C. See patent family annex.

Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

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